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Structural insights into selectivity and cofactor binding in snake venom L-amino acid oxidases

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ABSTRACT

L-Amino acid oxidases (LAAOs) are flavoenzymes that catalytically deaminate L-amino acids to corresponding α-keto acids with the concomitant production of ammonia (NH_3) and hydrogen peroxide (H_2O_2). Particularly, snake venom LAAOs have been attracted much attention due to their diverse clinical and biological effects, interfering on human coagulation factors and being cytotoxic against some pathogenic bacteria and *Leishmania* ssp. In this work, a new LAAO from *Bothrops jararacussu* venom (BjsuLAAO) was purified, functionally characterized and its structure determined by X-ray crystallography at 3.1 Å resolution. BjsuLAAO showed high catalytic specificity for aromatic and aliphatic large side-chain amino acids. Comparative structural analysis with prokaryotic LAAOs, which exhibit low specificity, indicates the importance of the active-site volume in modulating enzyme selectivity. Surprisingly, the flavin adenine dinucleotide (FAD) cofactor was found in a different orientation canonically described for both prokaryotic and eukaryotic LAAOs. In this new conformational state, the adenosyl group is flipped towards the 62–71 loop, being stabilized by several hydrogen-bond interactions, which is equally stable to the classical binding mode.

1. Introduction

A number of proteins, enzymes and biologically active peptides that interfere with key physiological processes are present in snake venoms, triggering a wide spectrum of secondary effects such as blood clotting, myotoxicity, neurotoxicity, platelet aggregation and lipid digestion [1,2]. Proteins that act at specific points and interfere with the highly-regulated blood coagulation cascade and platelet aggregation have been recruited to serve as diagnostic and clinical tools [3,4].

L-Amino acid oxidases (LAAOs; E.C. 1.4.3.2) are flavoenzymes that catalytically deaminate L-amino acids to the corresponding α-keto acids with the production of ammonia (NH_3) and hydrogen peroxide (H_2O_2) [5,6]. LAAOs are cytotoxic proteins [7,8], which inhibit platelet aggregation [8,9] and are active against *Leishmania* spp. [10], bacteria, and viral proteins [5–11]. Since the bactericidal activity of LAAO is inhibited by catalase, this suggests that hydrogen peroxide is important in these processes [11].

LAAOs can be inactivated by decreasing the pH and can be reactivated by increasing pH [12] and inactivation by freezing is more pronounced between −20 and −30 °C [13]. These results suggest that inactivation of the enzyme is likely due to conformational changes in the protein structure, particularly around the flavin binding site [13].

Although their content varies between genera and species, most of the Viperidae venoms contain LAAOs. In *Bothrops* species, LAAOs represent approximately 2% of the total weight of the desiccated crude venom and leads to the typical yellow color [10,11]. Although LAAOs have been isolated from different organisms, snake venom LAAOs are the best characterized [7]; however, there is as yet no clear correlation between their structural and toxic properties. Here we present the results of the purification and structural characterization of a new L-amino acid oxidase from the venom of *Bothrops jararacussu* (BjsuLAAO) to improve this correlation.

2. Materials and methods

2.1. Two-step purification procedure

Desiccated crude venom (125 mg) was suspended in 1.5 ml of 0.02 M Tris–HCl buffer containing 0.15 M NaCl, pH 8.0 and centrifuged at 10,000×g for 10 min. The clear supernatant (1 ml) was applied onto a 16/60 Sephacryl S-100 column previously equilibrated with the aforementioned buffer. The protein fractions were eluted

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at a flow rate of 0.2 ml/min and fractions of 1 ml/tube were collected, the absorption was monitored at 280 nm and the fractions were analyzed by SDS–PAGE [14]. The fractions forming the second peak of size-exclusion chromatography (SEC) were pooled and were concentrated to 0.5 ml using a micro-concentrator (AMICON) with a 30 kDa membrane (Fig. S1) and applied onto a Mono Q 5/50 GL column. The column was washed with 0.02 M Tris–HCl buffer pH 8.0 (eluent A) and eluted with a nonlinear salt gradient (eluent A + 1 M NaCl). Protein concentration was determined according to a microbiuret method described by [15], using bovine serum albumin as standard.

2.2. Substrate specificity

Substrate specificity was determined by dissolving 2 mM of the amino acids L-Histidine, L-Glutamine, L-Threonine, L-Serine, L-Lysine, L-Arginine, L-Phenylalanine, L-Tryptophan, L-Leucine, L-Isoleucine, L-Methionine, L-Cystine, L-Cysteine, L-Valine and L-tyrosine in 500 μl of 0.1 M Tris–HCl pH 7.2 with the addition of 5 μl of solution containing *O*-phenylenediamine (OPD) (10 mg/ml) and 1 μl of peroxidase (1 mg/ml). The final solution was incubated with 3 μg of enzyme for 30 min at 25 °C. The reaction was blocked with 500 μl of 10% citric acid (w/v) and the absorbance was determined at 490 nm.

2.3. Crystallization

The purified LAAO from *B. jararacussu* (BjsuLAAO) was concentrated to 9 mg/ml in micro-concentrators (AMICON MWC 30 kDa) and stored in a 0.02 M Tris–HCl pH 8.0 buffer at 4 °C. Crystallization was performed by the hanging-drop vapor-diffusion method using 24-well tissue-culture plates [16] and commercially available crystallization screens such as crystal screen 1 and 2, polyethylene glycol 6000, ammonium sulfate kits (Hampton research) and the PEG suite (Quiagen). Typically, 1 μl of a protein solution was mixed with an equal volume of the screening solution and equilibrated over a reservoir containing 0.5 ml of the latter solution. Crystals suitable for diffraction were obtained with 0.1 M sodium acetate trihydrate pH 4.6 and 25% (w/v) polyethylene glycol 1000.

2.4. Data collection and structure determination

A BjsuLAAO crystal was directly flash-cooled in a 100 K nitrogen-gas stream and X-ray diffraction data were collected on the W01B-MX2 beamline at the Brazilian Synchrotron Light Laboratory (Campinas, Brazil). The wavelength of the radiation source was set to 1.458 Å and a MarMosaic 225 mm CCD detector was used to record the X-ray diffraction intensities. The data were indexed, integrated and scaled using the DENZO and SCALEPACK programs from the HKL-2000 package [17]. Molecular replacement was carried out using the MOLREP program [18] and a model based on the atomic coordinates of native L-amino acid oxidase from *Vipera amodytes amodytes* (PDB code 3KVE) [2].

3. Results and discussion

BjsuLAAO was isolated from the *B. jararacussu* venom by two chromatographic steps (SEC and AEC) yielding 5 mg of purified enzyme from an initial amount of 250 mg of crude venom (Figs. S1–S4).

3.1. BjsuLAAO has higher specificity for hydrophobic residues

The BjsuLAAO activity was tested using different amino acids as substrate (Fig. 1). The enzyme showed high activity for aromatic and aliphatic amino acids with large side chain including L-Methionine, L-Leucine, L-Phenylalanine, L-Isoleucine, L-Tryptophan and L-Tyrosine. A significant activity was also observed for L-Cysteine. Over other amino acids, BjuLAAO had low catalytic activity (Fig. 1). This pattern shows a clear preference for hydrophobic residues with voluminous side chain and the affinity for residues with polar and/or small side-chains is significantly reduced or absent (Fig. 1). The LAAO from *Bothrops pauloensis* also showed preference for Met-, Leu-, Phe- and Ile- as substrates [7], suggesting that LAAOs encountered in *Bothrops* genus retain similar functions. In contrast, the LAAO from *Bungarus fasciatus* displayed higher specificity towards Tyr- and Asp- [19], whereas the bacterial LAAO (*Rhodococcus opacus*) has a very low substrate specificity hydrolyzing aromatic, aliphatic and polar amino acids [20].

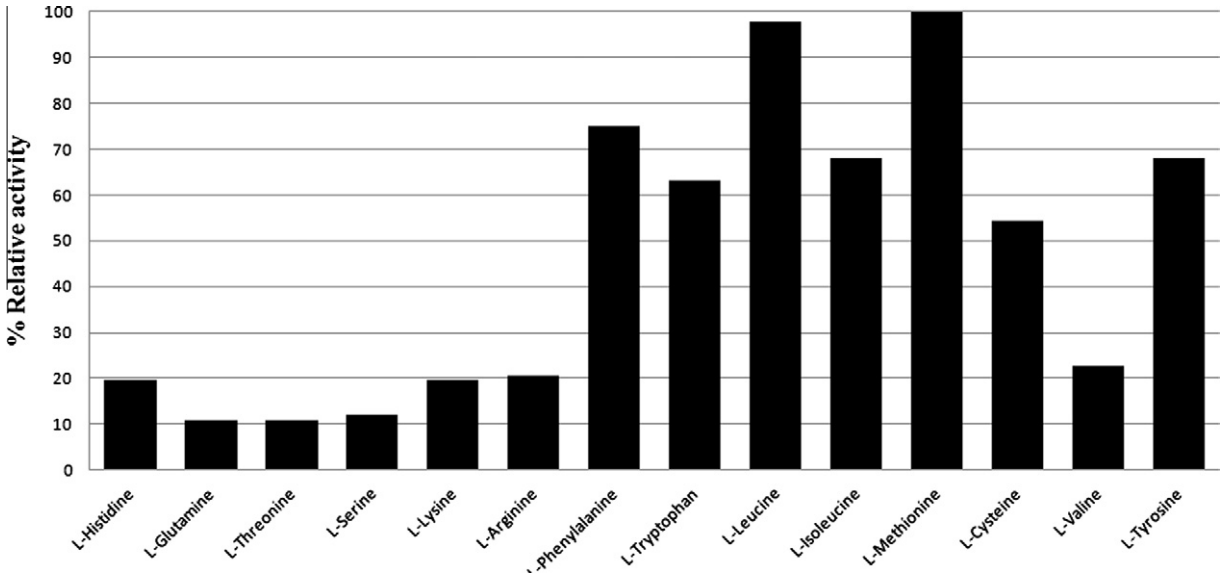

Fig. 1. Substrate specificity histogram. The LAAO activity was tested using different amino acids as substrate. We detected high activity for aromatic and hydrophobic amino acids, L-Methionine, L-Leucine, L-Phenylalanine, L-Isoleucine, L-Tryptophan and L-Tyrosine. The LAAO showed low catalytic activity for L-Histidine, L-Arginine, L-Valine, L-Threonine, L-Glutamine, L-Lysine and L-Serine.